Variation of Potential Nitrification and Ammonia-Oxidizing Bacterial Community with Plant-Growing Period in Apple Orchard Soil

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Abstract

In this study, we investigated the potential nitrification and community structure of soil-based ammonia-oxidizing bacteria (AOB) in apple orchard soil during different growth periods and explored the effects of environmental factors on nitrification activity and AOB community composition in the soil of a Hanfu apple orchard, using a culture-dependent technique and denaturing gradient gel electrophoresis (DGGE). We observed that nitrification activity and AOB abundance were the highest in November, lower in May, and the lowest in July. The results of statistical analysis indicated that total nitrogen (N) content, NH₄⁺-N content, NO₃⁻-N content, and pH showed significant correlations with AOB abundance and nitrification activity in soil. The Shannon-Winner diversity, as well as species richness and evenness indices (determined by PCR-DGGE banding patterns) in soil samples were the highest in September, but the lowest in July, when compared to additional sampled dates. The DGGE fingerprints of soil-based 16S rRNA genes in November were apparently distinct from those observed in May, July, and September, possessing the lowest species richness indices and the highest dominance indices among all four growth periods. Fourteen DGGE bands were excised for sequencing. The resulting analysis indicated that all AOB communities belonged to the β-Proteobacteria phylum, with the dominant AOB showing high similarity to the Nitrosospira genus. Therefore, soil-based environmental factors, such as pH variation and content of NH₄⁺-N and NO₃⁻-N, can substantially influence the abundance of AOB communities in soil, and play a critical role in soil-based nitrification kinetics.

Key words: apple orchard soil, ammonia-oxidizing bacteria, potential nitrification, community structure, PCR-denaturing gradient gel electrophoresis

INTRODUCTION

The species diversity of soil-based ecosystems are commonly limited under nitrogen (N)-limiting conditions, with the transformation of N-based compounds from one form into another being regulated in a tightly coupled cycle (Rennenberg et al. 2009; Shen et al. 2012). Nitrogen limitation is exacerbated during the rapid growth stage of fruit tree and in harvested soils, where the N content in the biomass has been removed, and subsequent leaching, erosion, and increased N cycling rates further deplete nutrient availability (Grigal et al. 2000; Chanasyk
et al. 2003; Miao et al. 2011). Nitrification is a major process in N cycling, and consists of a 2-step process: the oxidation of ammonia (NH₃) to nitrite (NO₂⁻) and, subsequently, nitrite to nitrate (NO₃⁻) (Prosser 1989). Chemolithotrophic ammonia-oxidizing bacteria (AOB) are aerobic, obligate autotrophs (Wrage et al. 2001) that utilize NH₃ as their sole electron acceptor source for respiration and carbon dioxide as their primary carbon source (Kowalchuk and Stephen 2001). AOB are responsible for the rate-limiting step in nitrification in a wide variety of environments, and, as such, are crucial in the global N cycle (Kowalchuk et al. 1997; Kowalchuk and Stephen 2001; Shen et al. 2011).

China is now the world’s largest producer, consumer and importer of chemical fertilizers. Over application of nitrogen-based fertilizer is common in intensive agricultural regions, and current N-uptake efficiency has been reported to be less than 20% for fruit trees (Miao et al. 2011). In addition to surface and groundwater pollution and greenhouse gas emissions, the overapplication of N fertilizers has resulted in significant soil acidification in major Chinese orchard systems, posing a great challenge for fruit security of apple production in Jiaodong Peninsula and other traditional apple orchard regions in China. For instance, soil acidification increases the susceptibility of apple trees to rough bark disease and bitter pit disease, leading to a decline in apple yield and quality. From an agricultural perspective, nitrification represents a potential source of N loss (through which N-based fertilizer becomes unavailable for plant nutrition) and a source of greenhouse gas emission through the generation of nitrous oxide (N₂O) gas (Kong et al. 2010). Therefore, research on nitrification has caused much attention since the 1980s due to intensified application of ammonia-based N-based fertilizers and their associated environmental consequences (Holly et al. 2012). Moreover, AOB has long been proposed to play important roles in the soil nitrogen cycle, thereby affecting the utilization efficiency of soil nitrogen by plant. Current studies have identified a number of environmental factors that may influence the composition of the AOB community. Bäckman et al. (2004) reported that the change in AOB community structure was driven by ammonium (NH₄⁺) availability and potential nitrification associated with harvesting disturbances. A wide variety of environmental factors have been linked to AOB community structure, including soil NH₄⁺ content (Hastings et al. 1997; Mintie et al. 2003), potential nitrification activity (Bäckman et al. 2004), vegetation cover (Boyle-Yarwood et al. 2008), temperature (Avrahami and Conrad 2005), pH (Bäckman et al. 2003; Yeager et al. 2005), and carbon-to-nitrogen (C/N) ratio (Nugroho et al. 2005). Diversity in the types of N-based fertilizers applied may influence the AOB community structure and corresponding nitrification. Tong and Xu (2012) reported that the addition of urea stimulated the AOB population size, thereby accelerating nitrification and soil acidification, while addition of (NH₄)₂SO₄ inhibited the growth of AOB communities and had a negative impact on nitrification.

Molecular techniques offer a powerful means to investigate AOB community composition in response to environmental changes. By separating unique genetic sequences in a polyacrylamide gel based on nucleotide composition, polymerase chain reaction coupled with denaturing gel gradient electrophoresis (PCR-DGGE) is widely used to assess microbial community changes in the soil ecosystem (Muyzer et al. 1993; Nicolaisen and Ramsing 2002; dos Santos et al. 2012).

Studies on the ecological distribution and community dynamics of AOB across a wide range of soil habitats and management regimes worldwide have demonstrated broad physiological diversity and distinct ecophysiology under contrasting soil and climatic conditions. However, it remains unknown whether a diversity of functions, or possible functional redundancy, of phylogenetically distinct AOB groups exist under varying environmental conditions. In order to reduce nutrient loss and to gain improved understanding of the microbial communities involved, as well as their specific contributions to nitrification and soil N-cycling, further research on temporal variations of AOB activity and their unique contributions to nitrification is required, particularly in apple orchard soils, where nitrogen saturation occurs with increasing NO₃⁻-N leaching, by excess use of N-based fertilizer annually to enhance crop.
production. The development of improved N-cycling management strategies to optimize and improve the efficiency of N utilization in an ecologically and environmentally friendly manner would be highly beneficial. Therefore, the objective of this study was to investigate a possible linkage between AOB community shifts and soil-based physicochemical property changes in different growth periods, and to determine the relationship between AOB community composition and nitrification activity in apple orchard soil.

RESULTS

Physicochemical parameters analysis

Soil physicochemical properties varied greatly among the four phenological stages of apple orchard development (November, May, July, and September), except for the C/N ratio (Table 1). Specifically, soil samples from July had the highest total C content, total N content, organic matter, available P content, NH$_4^+$ -N content, and NO$_3^-$ -N content, but the lowest pH and C/N ratio, compared to the soil samples from the remaining three periods. Most of the soil parameters were lower in November than in May, July and September, but the pH values were the highest in November.

The correlation of AOB most probable number with environmental variables

The most probable number (MPN) showed its lowest and highest values in July and November, respectively, which correlated with 110.0 cells g$^{-1}$ and 2000 cells g$^{-1}$ fresh soil, respectively (Fig. 1). There were no obvious differences between the orchard soils sampled in May and November.

In orchard soil, the MPN was negatively correlated to total C content ($r=-0.888$, $P<0.01$), total N content ($r=-0.929$, $P<0.01$), NH$_4^+$ -N content ($r=-0.836$, $P<0.01$), organic substrate content ($r=-0.824$, $P<0.01$), and available P content ($r=-0.644$, $P<0.05$), but positively correlated to soil pH ($r=0.932$, $P<0.001$) (Table 2).

The correlation of potential nitrification activity with environmental variables

In apple orchard soil, the potential nitrification (PN) rates showed a similar trend with MPN during different growth periods, with soil samples from November displaying the highest PN rates and those from July displaying the lowest rates. However, no significant differences ($P>0.05$) were detected between the soil samples from May and September.

The correlation analysis indicated that total N

<p>| Table 1 | Physicochemical properties of apple orchard soil sampled during four phenological periods |</p>
<table>
<thead>
<tr>
<th>Sampled time (mon-d)</th>
<th>Total C (%)</th>
<th>Total N (N%)</th>
<th>C/N ratio</th>
<th>Organic matter (mg kg$^{-1}$)</th>
<th>Available P (mg kg$^{-1}$)</th>
<th>Available K (mg kg$^{-1}$)</th>
<th>Water content (%)</th>
<th>NH$_4^+$ -N (mg kg$^{-1}$)</th>
<th>NO$_3^-$ -N (mg kg$^{-1}$)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>05-05</td>
<td>1.19±0.09 c</td>
<td>0.15±0.01 c</td>
<td>7.56±0.57 a</td>
<td>21.52±2.56 c</td>
<td>8.93±4.32 b</td>
<td>34.24±20.91 a</td>
<td>19.97±1.67 a</td>
<td>0.48±0.03 b</td>
<td>46.39±6.82 b</td>
<td>6.33±0.02 b</td>
</tr>
<tr>
<td>07-01</td>
<td>1.76±0.02 a</td>
<td>0.24±0.01 a</td>
<td>7.28±0.06 a</td>
<td>32.34±1.75 a</td>
<td>258.87±13.22 b</td>
<td>14.77±1.74 b</td>
<td>1.83±0.03 a</td>
<td>55.7±1.41 a</td>
<td>5.50±0.05 d</td>
<td></td>
</tr>
<tr>
<td>09-09</td>
<td>1.40±0.02 b</td>
<td>0.18±0.01 b</td>
<td>7.48±0.49 a</td>
<td>28.28±0.90 ab</td>
<td>234.87±19.28 bc</td>
<td>10.06±1.46 c</td>
<td>0.49±0.04 b</td>
<td>26.79±4.40 c</td>
<td>6.17±0.04 c</td>
<td></td>
</tr>
<tr>
<td>11-12</td>
<td>1.34±0.02 b</td>
<td>0.16±0.01 c</td>
<td>7.93±0.27 a</td>
<td>25.34±2.41 b</td>
<td>46.79±3.04 d</td>
<td>225.30±7.95 c</td>
<td>8.00±1.64 c</td>
<td>0.43±0.02 b</td>
<td>26.54±1.00 c</td>
<td>6.47±0.05 a</td>
</tr>
</tbody>
</table>

Means±standard deviation (n=4) values indicated by the same letter within a row are not significantly different at $P<0.05$ level according Duncan’s test.

<p>| Table 2 | Pearson’s correlation coefficients between the soil physicochemical properties and soil PN activity, MPN, and AOB diversity |</p>
<table>
<thead>
<tr>
<th>Item$^1$</th>
<th>PN</th>
<th>MPN number</th>
<th>Total N</th>
<th>Total C</th>
<th>Organic matter</th>
<th>AP</th>
<th>AK</th>
<th>Water content</th>
<th>pH</th>
<th>NH$_4^+$ -N</th>
<th>NO$_3^-$ -N</th>
<th>C/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN</td>
<td>1.000</td>
<td>0.888</td>
<td>0.888</td>
<td>-0.929</td>
<td>-0.888</td>
<td>-0.824</td>
<td>-0.644</td>
<td>0.215</td>
<td>0.932</td>
<td>-0.836</td>
<td>-0.415</td>
<td>0.516</td>
</tr>
<tr>
<td>MPN number</td>
<td>0.888$^*$</td>
<td>1.000</td>
<td>-0.929$^*$</td>
<td>-0.888$^*$</td>
<td>-0.824$^*$</td>
<td>-0.644</td>
<td>-0.877$^*$</td>
<td>-0.583$^*$</td>
<td>-0.697$^*$</td>
<td>-0.959$^*$</td>
<td>-0.590$^*$</td>
<td>0.131</td>
</tr>
<tr>
<td>S</td>
<td>-0.059</td>
<td>-0.022</td>
<td>-0.303</td>
<td>-0.374</td>
<td>-0.142</td>
<td>-0.375</td>
<td>0.074</td>
<td>0.038</td>
<td>0.264</td>
<td>-0.487</td>
<td>-0.371</td>
<td>-0.165</td>
</tr>
<tr>
<td>D</td>
<td>0.137</td>
<td>-0.316</td>
<td>0.345</td>
<td>0.439</td>
<td>0.616</td>
<td>-0.391</td>
<td>-0.955</td>
<td>-0.484</td>
<td>-0.115</td>
<td>-0.09</td>
<td>-0.590</td>
<td>0.131</td>
</tr>
<tr>
<td>H</td>
<td>0.326</td>
<td>0.130</td>
<td>-0.367</td>
<td>-0.359</td>
<td>-0.035</td>
<td>-0.765</td>
<td>-0.425</td>
<td>-0.510</td>
<td>0.469</td>
<td>-0.639</td>
<td>-0.802</td>
<td>0.159</td>
</tr>
<tr>
<td>J</td>
<td>0.504</td>
<td>0.235</td>
<td>-0.398</td>
<td>-0.353</td>
<td>-0.027</td>
<td>-0.877</td>
<td>-0.583</td>
<td>-0.697</td>
<td>0.551</td>
<td>-0.670</td>
<td>-0.915</td>
<td>0.285</td>
</tr>
</tbody>
</table>

$^1$PN, potential nitrification; MPN, most probable number; S, Richness index; D, Dominance index; H, Shannon-Winner index; J, Evenness index.

$^*$AP, available P; AK, available K.

$^*$and $^*$, correlation is significant at the 0.05 and 0.01 probability levels, respectively.
content \((r=0.788, P<0.01)\), available P content \((r=0.843, P<0.01)\), total C content \((r=-0.698, P<0.05)\), NH\(_4^+\)-N content \((r=-0.814, P<0.01)\), and NO\(_3^-\)-N content \((r=-0.692, P<0.05)\) were associated with significant reductions in soil-based potential nitrification values, whereas pH \((r=0.902, P<0.001)\) and C/N ratio \((r=0.609, P<0.05)\) were increased. Specifically, the potential nitrification was highly correlated with MPN \((r=0.888, P<0.01)\) in the apple orchard soil (Table 2).

The correlation of AOB community diversity with environmental variables

The DGGE banding patterns revealed substantial shifts of AOB composition along different growth periods (Fig. 2). The measured indices were Shannon-Winner \((H)\), Richness \((S)\), Evenness \((J)\), and Dominance \((D)\). Among the four periods, soil samples from July demonstrated the lowest \(H\), \(S\) and \(J\) values (Table 3), whereas soil samples from September demonstrated the highest values. Although soil samples from November demonstrated the second highest \(H\) and \(J\) values (following the samples from September), they were identical to soil samples from July in demonstrating the lowest \(S\) value, but the highest \(D\) value among all four sampled periods.

Differences in the Pearson’s correlation coefficients between soil characteristics and AOB diversity displayed in Table 2 indicate that the \(H\), \(J\) and \(D\) indices were significantly depressed by water content, available P and K content, as well as NH\(_4^+\)-N and NO\(_3^-\)-N content in soil. Specifically, (1) the \(H\) index displayed significant correlations to available P content \((r=-0.765, P<0.01)\), NH\(_4^+\)-N content \((r=-0.639, P<0.05)\), and NO\(_3^-\)-N content \((r=-0.802, P<0.01)\); (2) the \(D\) index displayed significant correlations to available K content \((r=0.955, P<0.01)\), water content \((r=0.848, P<0.01)\), and NO\(_3^-\)-N \((r=0.590, P<0.05)\); and (3) \(J\) index displayed significant correlations to available P content \((r=0.955, P<0.01)\), available K content \((r=0.583, P<0.05)\), water content \((r=0.697, P<0.05)\), NH\(_4^+\)-N content \((r=0.670, P<0.05)\), and NO\(_3^-\)-N content \((r=0.915, P<0.01)\). By contrast, no significant correlation was observed among AOB

**Table 3** AOB community diversity at different growth stages of Hanfu apple orchard soil

<table>
<thead>
<tr>
<th>Sample time (mon-d)</th>
<th>Shannon-Winner index ((H))</th>
<th>Richness index ((S))</th>
<th>Evenness index ((J))</th>
<th>Dominance index ((D))</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-5</td>
<td>0.422</td>
<td>16</td>
<td>0.351</td>
<td>9.324</td>
</tr>
<tr>
<td>7-1</td>
<td>0.359</td>
<td>13</td>
<td>0.323</td>
<td>15.486</td>
</tr>
<tr>
<td>9-9</td>
<td>0.617</td>
<td>20</td>
<td>0.474</td>
<td>17.950</td>
</tr>
<tr>
<td>11-12</td>
<td>0.469</td>
<td>13</td>
<td>0.421</td>
<td>18.130</td>
</tr>
</tbody>
</table>

Fig. 1 AOB MPN and nitrification activity at different growth stages of Hanfu apple orchard soil.

Fig. 2 DGGE fingerprints of 16S rRNA genes of AOB communities at different growth stages of Hanfu apple orchard soil.
diversity and pH value, total C content, total N content, or organic matter. Although the nutrients typically have a positive effect on the growth of soil bacteria, the significant negative correlations observed in the present study indicate that key environmental variables, such as pH, total N content, available P content, NH$_4^+$-N content, and NO$_3^-$-N content, have a stronger impact on the AOB growth, which generally displayed a weaker correlation with other soil nutrients (Li et al. 2011).

AOB community composition

To further confirm variability in the AOB composition at different time periods, 14 dominant DGGE bands with high intensities on the DGGE gel were selected for sequencing. Using BLAST, we compared the sequences generated with the database and found that the majority of sequences exhibited similarities ranging from 98-100% (Fig. 3). Sequence analysis by the BLAST algorithm revealed that the entire AOB present in this study belonged to the phylum β-Proteobacteria. Most of these AOB sequences were derived from uncultured AOB strains, and the dominant AOB communities displayed high similarity to the genus *Nitrosospira*, accounting for 21.4% of the sequenced bands. However, several non-AOB sequences, including *Methylophilus*, *Nitrospira* and *Dechloromonas*, were also found in apple orchard soils.

Among the analyzed DGGE bands, band 4 was affiliated with the uncultured β-Proteobacteria and displayed higher intensity than other bands did in DGGE profiles, particularly in the samples from November (Fig. 2). This characteristic band may play an important role in enhancing soil nitrification activity. Additionally, bands 2 and 3 in soil samples from November were brighter than those in soil samples from other growth periods. Bands 9 and 13 were only apparent in soil samples from November and May, respectively, implying that several AOB communities may have adapted to environmental changes and assisted in the enhancement of soil PN in apple orchard soil. Taken together, these results indicated that the AOB community structure differed during the four growth periods, and the predominant

Fig. 3 Neighbor-joining tree depicting the phylogenetic relationships among the AOB at different growth stages of Hanfu apple orchard soil.
AOB in apple orchard soils may play an important role in the MPN and the function in nitrogen cycling of soil.

DISCUSSION

Soil pH is known to have a considerable effect on the activities of microbial communities and the biogeochemical processes they mediate. Soil pH will affect the chemical form, concentration and availability of substrates in the local soil area (Allison and Prosser 1993; Kemmitt et al. 2006) and will mediate cell growth and activity. There also exists strong evidence that soil pH is an important determinant of bacterial diversity and community structure at a global scale (Fierer and Jackson 2006). Ammonia oxidation in soil is substantially reduced in acidic soils (de Boer and Kowalchuk 2001; Tong et al. 2012), and significant batch growth of pure AOB cultures in liquid growth media does not occur below pH 7 (de Boer and Laanbroek 1989; Allison and Prosser 1991; Jiang and Bakken 1999) due to the insufficient energy obtained for growth (Allison and Prosser 1993). The results of this study showed that the pH values of both the PN and MPN in apple orchard soil were higher in November (pH 6.47) and May (pH 6.33), but lower in July (pH 5.50). Potential nitrification and MPN had a significant positive correlation with soil pH, suggesting that soil pH could affect both the AOB populations and subsequent nitrification activity in apple orchard soil. Generally accepted explanation for reduced growth and activity of ammonia oxidizers at low pH is the exponential reduction in NH₃ availability with decreasing pH due to its ionization to NH₄⁺ (Frijlink et al. 1992). This reaction may lead to the decline in the quantity of NH₃, diffused into cells, where substantially higher energy is required for intracellular NH₄⁺ transport. The quantity of bacterial transcripts increased with increasing pH up to a maximum of 6.9 (Shen et al. 2012). This observation is consistent with results of autotrophic nitrification performed by Killham (1990), where the maximum rates of nitrification occurred in pH 6.5 plots.

It was confirmed that the roles of AOB and ammonia-oxidizing archaea (AOA) in soil ammonia oxidation varied under differing soil conditions, and were supposed to occupy different ecological niches based on N input and pH variation (He et al. 2012). Di (2009) and Xia (2011) reported that AOB may actively be implicated in the nitrification of alkaline and N-rich neutral soils. Shen (2011) observed that N-based fertilizer amendment altered the abundance and composition of AOB in a semi-arid temperate grassland soil with neutral pH. Since the period from blossoming to young shoot growth is characterized by intensive nitrogen intake for fruit trees and the post-harvest period is marked by nitrogen reserve, N-based fertilizer was typically applied during the two periods to meet the corresponding requirements. During the blossom (May) and post-harvest nutrient storage period (November), the need for nitrogen nutrition can accelerate the soil nitrogen transformation. This acceleration is achieved through AOB to meet the requirements of apple growth and development during a specific period and causes the promotion of soil nitrification. However, the total N content in the soil significantly decreased the PN and MPN in our study. This observation indicates that proper N content and neutral soil pH are beneficial for N transformation and supply, as well as for the enhancement of N-based fertilizer efficiency in apple orchard soil. Thus, changes in N content, especially the NH₄⁺-N and NO₃⁻-N content, as well as pH, in the orchard soils from different periods paralleled the nitrification and MPN, suggesting that N content and pH may represent important variables affecting AOB populations and their nitrification activities.

The PCR-DGGE profile of bacterial ammonia oxidizers showed that AOB diversity varied widely in apple orchard soil during different growth periods. The diversity, richness and evenness indices were lowest in soil samples from July (Table 3) compared with other sampling dates. This observation may be attributed to increased soil acidification resulting from plentiful rains during the season and the low potential nitrification observed in July. The AOB community diversity was inhibited at low pH, thereby ultimately reducing AOB function. These results were consistent with the findings reported by Nicol (2008), showing that AOB community diversity and corresponding ammonia
monooxygenase (amoA) gene expression, as well as soil potential nitrification, would be substantially inhibited in acidified soil.

Soil-based AOB community composition changes with varying temperatures and rhizosphere physicochemical properties over the course of plant growth, which affects the process kinetics of soil nitrogen transformation and, through the succession of community diversity, the enhancement of nitrification activity (Hermansson et al. 2004). The results of this study indicated that the soil-based AOB community structure was diversified during different growth periods, resulting in differences in MPN and soil nitrification potential. Shen (2012) reported that the AOB community was primarily dominated by *Nitrosospira* and *Nitrosomonas*, which are frequently detected in acidic, alkaline and neutral soils. Although *Nitrosospira* was found to be the dominant AOB genus in apple orchard soil in this study, *Methylophilus*, *Nitrosirpa* and *Dechloromonas*, which were not AOB communities, were also observed in our study. These results may be attributed to the susceptibility of PCR-based techniques of introducing biases associated with cell lysis, nucleic acid extraction, choice of primer, and amplification errors (Mathieu-Daudé et al. 1996; Lueders and Friedrich 2003). In addition, CTO primers, which are selective for known betaproteobacterial AOB, typically amplify non-AOB sequences when AOB are present at low relative abundance (Bäckman et al. 2003; Cebron et al. 2004), introducing a bias that might be markedly amplified using universal 341f-518r primer for the second round of PCR performed in our study. In the meantime, the CTO primer set is generally considered to be biased toward the *Nitrosospira* lineage (Purkhold et al. 2000) and may limit AOB diversity studies in a variety of environments to an extent (Koops and Pommerening-Röser 2001). Thus, the sequence biases due to nonspecific amplification by CTO primers may account for the appearance of non-AOB sequences in our study. Mahmoud et al. (2006) demonstrated that the correct choice of primer strategy was important, and nested amplification with universal bacterial 27f-1492r primers, followed by CTO189f-CTO654r primers, may help reduce sequencing biases observed in the characterization of ammonia-oxidizing communities in soil. Therefore, the non-AOB sequences obtained in apple orchard soil require further examination, and selecting the appropriate primer strategies and screening for several approaches in investigation of AOB communities should be performed in future studies.

Soil environmental factors, such as ammonia availability, temperature, acidity, and other variables can influence the AOB community structure, abundance, and geographic distribution (Di et al. 2009; Malchair et al. 2010). In our study, soil nitrification potentials and AOB MPN were affected by soil-based total nitrogen content, pH, NH$_4^+$-N content, NO$_3^-$-N content, and other environmental factors. AOB community diversity and evenness and dominance indices all exhibited substantial relevance to environmental factors, including NH$_4^+$-N content, NO$_3^-$-N content, available phosphorus content, potassium content, and water content, to varying extents. The increase of the dominance index in November further showed that orchard soil-based AOB adapted to soil-based environmental changes by adjusting their community structure and population size. They provided the corresponding soil nitrogen nutrients to support apple tree growth by promoting the soil nitrification process. However, the mechanism by which these AOB respond to various biotic and abiotic variations, as well as their distribution and functional differences in varying soil systems remains unclear and requires further investigation.

**CONCLUSION**

The AOB abundances were positively correlated to soil nitrification in apple orchard. The differences in soil pH value and total N content, available P content, NH$_4^+$-N content, and NO$_3^-$-N content in soil in different growth periods are important factors affecting the AOB community structure and the potential nitrification of apple orchard soil.

The dominant AOB community in apple orchard soil was *Nitrosospira*. Additionally, AOB composition and population differed during the various growth periods, which may affect the final potential nitrification of the soil.
MATERIALS AND METHODS

Site description and soil sampling methodology

The apple orchard investigated in this study is located at Suizhong (119°49'E, 40°08'N; 90 m altitude), south of Liaoning Province, China. The area of investigation is an important apple production region in Liaoning Province with a mean temperature of 9.1°C that receives annual rainfall of 645 mm. The predominant soil type is brown soil. The soil physicochemical properties were analyzed, with the general description listed in Table 1.

Soil samples were collected in 2011 from the top 0-20 cm soil depth of the apple orchard, including four phenological time points, May 5 (blossom period), July 1 (young shoot-growing period), September 9 (fruit-enlargement period), and November 12 (defoliation). The sample site was situated under the tree, at a distance of 0.5 m from the trunk. Four sampling points were used at each site. 1 kg samples were collected at each sampling site for physicochemical and AOB community analysis.

Soil samples were stored on ice until they were returned to the laboratory where they were sieved with a <2-mm mesh and stored at 4°C. One set of subsamples was air-dried and pulverized for general soil characterization, and a second set was stored at -80°C for subsequent DNA extraction and amplification.

Physicochemical characterization of soil samples

All soil analyses were performed based on Hart et al. (1994). Gravimetric water content (GWC) was determined by calculating the mass lost after drying a known quantity of soil at 105°C for 48 h, and expressed as mass of water per unit mass of dry soil (%). Soil pH was measured in a 2:1 soil:double-deionized water slurry. Total C and total N content were quantified by the combustion method using a LECO CNS-2000 (LECO Cooperation, USA) and expressed as a percentage of the total soil mass (Helgason et al. 2009). The carbon-to-nitrogen (C/N) ratio was calculated by dividing the total C content by the total N content (unitless). NH$_4^+$-N was extracted with 1 mol L$^{-1}$ KCl (1:5, w:v) and analyzed colorimetrically using a continuous flow analyzer (Autoanalyser; Bran+Luebbe, Germany). Nitrification rates were calculated by linear regression of nitrate concentrations over time ($\mu$g N g$^{-1}$ h$^{-1}$).

Total DNA was extracted from 0.5 g of orchard soil using the Fast DNA Spin Kit for soil (MP Biomedicals, Canada). Cell lysis was performed by vigorous shaking using a bead beater according to the manufacturer’s instructions. The quality of the extracted DNA was evaluated by electrophoresis on a 0.8% agarose gel and the DNA was visualized on a Bio-Rad Gel Doc XR System with Image Lab Software (Bio-Rad, Canada).

PCR amplification

16S rRNA genes were amplified from extracted DNA using a nested PCR approach (Freitag et al. 2006). CTO189f and CTO654r PCR primers were used for primary amplification (Kowalchuk et al. 1997), and amplicons were nested with P3 (357f-GC) and P2 (518r) primers (Muyzer et al. 1993). Cycling conditions for amplifying AOB were 95°C for 5 min; followed by 10 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min; followed by 72°C at 10 min (Nicol et al. 2008). PCR product was confirmed on a 1% agarose gel followed by staining with GeneFinder to confirm their sizes.

DGGE analysis and sequencing of excised bands

DGGE was performed using 8% acrylamide gel with a 45-60% denaturant gradient, where 100% denaturant was defined as 7 mol L$^{-1}$ urea plus 40% formamide. The electrophoresis was conducted with the DCode System (Bio-Rad, USA) at 60°C, 180 V, 6 h. The gel was stained for 30 min with GeneFinder before visualization by a Bio-Rad Gel Doc XR System with Image Lab Software (Bio-Rad, Canada). Dominant bands were excised and eluted DNA was re-amplified using the aforementioned primers (P3 and P2)
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and PCR cycle program. Finally, re-amplified DNA with the primers (P3 and P2 without GC claps) from the DGGE bands were sequenced by Invitrogen Life Technologies (Shanghai, China) and submitted to the GenBank database to confirm AOB origins (NCBI, Bethesda, USA).

Statistical analysis

Band detection and gel analysis of DGGE images were performed by Image software (Bio-Rad, Canada). The phylogenetic tree of 16S rDNA sequences was performed using molecular evolutionary genetic analysis (MEGA 4.0) software (Tamura et al. 2007). Multiple comparisons of means were performed by a Duncan’s test and the relationships between variables were examined using Pearson’s correlation coefficients.

Acknowledgements

We would like to thank the National Natural Science Foundation of China (31101504 and 31171917), the Postdoctoral Science Foundation of China (2011M500575), the China Agricultural Research System (CARS-28), and the Shenyang Municipal Science and Technology Research Projects, China (F12-109-3-00) for their financial support.

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